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Characterization of the fast GABAergic inhibitory action of etifoxine during spinal nociceptive processing in male rats

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ABSTRACT

Etifoxine (EFX) is a non-benzodiazepine anxiolytic which potentiate $GABA_A$ receptor ($GABA_AR$) function directly or indirectly via the production of 3α-reduced neurosteroids. The later effect is now recognized to account for the long-term reduction of pain symptoms in various neuropathic and inflammatory pain models. In the present study, we characterized the acute antinociceptive properties of EFX during spinal pain processing in naive and monoarthritic rats using in vivo electrophysiology. The topical application of EFX on lumbar spinal cord segment, at concentrations higher than 30 μM, reduced the excitability of wide dynamic range neurons receiving non-nociceptive and nociceptive inputs. Windup discharge resulting from the repetitive stimulation of the peripheral receptive field, and recognized as a short-term plastic process seen in central nociceptive sensitization, was significantly inhibited by EFX at these concentrations. In good agreement, mechanical nociceptive thresholds were also significantly increased following an acute intrathecal injection of EFX. The acute modulatory properties of EFX on spinal pain processing were never seen in the simultaneous presence of bicuculline. This result further confirmed EFX antinociception to result from the potentiation of spinal $GABA_A$ receptor function.

1. Introduction

Several mechanisms of spinal disinhibition associated with pathological pain states have recently been characterized and potentiation of GABAergic inhibitory controls in the spinal cord is thought to be an efficient strategy to limit or prevent pain symptoms (Zeilhofer et al., 2012b). Indeed, intrathecal injections of positive allosteric modulators of $GABA_A$ receptors ($GABA_ARs$) function, such as benzodiazepines or 3α-reduced neurosteroids, reduced pain responses in various animal models and in human pain states (Goodchild and Serrao, 1987; Serrao et al., 1992). Results from these studies have, however, been difficult to interpret because of sedative, anxiolytic, and rewarding properties of these compounds. To overcome this difficulty, a growing number of studies are now attempting to use subtype-selective benzodiazepines (Zeilhofer et al., 2012a). In the case of neurosteroids, we recently adopted an alternative strategy aimed at stimulating their endogenous production with translocator protein (TSPO) agonists (Rupprecht et al., 2010). This strategy already appeared to be efficient in some models of pain while using olesoxime (TRO19622), a cholesterol derivative with neuroprotective properties (Bordet et al., 2008), for example. In our laboratory, we demonstrated that 3α-reduced neurosteroids produced after TSPO stimulation with the benzoxazine etifoxine (EFX) were responsible for the long-lasting analgesic effects, seen in several animal models of neuropathic and inflammatory pain (Aouad et al., 2009, 2014a, 2014b). Analgesic mechanisms included amplification of $GABA_AR$-mediated transmission, protection from prostaglandin E2-induced glycinergic disinhibition, reduction of pro-inflammatory processes and maintenance of proper chloride gradients (Aouad et al., 2014b).

Apart from these long-term effects mediated by TSPO, little is known on the acute modulation of $GABA_AR$ function by EFX in the spinal cord and its impact in spinal pain processing. Etifoxine (EFX) is commercially-available as a non-benzodiazepine anxiolytic in several countries (Micallef et al., 2001; Nguyen et al., 2006; Servant et al., 1998) and exerts positive allosteric modulation of β2/β3-containing $GABA_ARs$ (Hamon et al., 2003; Schlichter et al., 2000; Verleye et al., 1999, 2001). These subunits are likely to constitute most of the $GABA_ARs$ since they are widely expressed in all laminas of the spinal cord in rodents (Bohlhalter et al., 1996; Paul et al., 2012) and human (Waldvogel et al., 2010). So far, the precise

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location of EFX binding site on β subunit is not known. It is, however, apparently not overlapping with specific sites for benzodiazepine and neurosteroids since the potentiating action of EFX is not altered after binding of the silent benzodiazepine site antagonist flumazenil or of the neurosteroid allopregnanolone (Verleye et al., 1999, 2001). Using freshly dissociated spinal cord neurons, potentiation of $GABA_AR$ currents was observed with low micromolar concentrations of EFX (Schlichter et al., 2000).

To characterize the acute action of EFX on spinal nociceptive processing, we recorded deep dorsal horn neurons (i.e. wide dynamic range neurons), integrating peripheral noxious and non-noxious informations, in anesthetized adult rats. Action potential (AP) discharges resulting from the activation of non-noxious and noxious sensory neurons were analyzed in protocols of acute nociceptive stimulation of the receptive field (RF) and during short-term potentiation of action potential discharge (windup) induced by repetitive stimulation, as previously published (Juif and Poisbeau, 2013). Complementary experiments on a model of knee monoarthritic have also been performed.

2. Material and methods

Male Sprague Dawley rats (250–350 g; Janvier, Le Genest St Isle, France) were used for this study. They were housed by group of 4 under standard conditions (room temperature [22 °C], 12–12 h light–dark cycle) with *ad libitum* access to food and water. All experiments were conducted in conformity with the recommendations of the European Union directive on animal experimentation (2010/63/EU adopted on September 22, 2010) and were evaluated by the regional ethic committee in charge of animal experimentation (CREMEAS, authorization AL 01/01/02/11). This study was conducted under the responsibility of authorized personnel (license 67-116 from the French Department of Agriculture to PP).

2.1. In vivo electrophysiology

Single unit extracellular recordings were made from dorsal horn neurons in the lumbar enlargement of the spinal cord of the rat following the procedure previously described elsewhere (Juif and Poisbeau, 2013). Briefly, a laminectomy was performed in anesthetized rats (isoflurane; Vaporizer Isotec 3 datex-Ohmeda) to expose the L4–L5 segments of the spinal cord. Before recordings, the cord was firmly attached by vertebral clamps and meninges were delicately removed, and the spinal cord surface was covered with a thin layer of mineral oil. Single-unit extracellular recordings were made with a stainless steel electrode (FK#02; FHC, UK) connected to a differential amplifier (DAM80, WPI). An electrode was lowered into the dorsal horn to record neurons located in the deep dorsal horn of the spinal cord. Data were acquired and analyzed by a CED 1401 analog-to-digital interface coupled to a computer with Spike 2 software (Cambridge Electronic Design, Cambridge, UK). All neurons included in the present study were wide dynamic range (WDR) neurons (Le Bars and Cadden, 2009) responding to both innocuous and noxious stimuli after electrical stimulation of the peripheral hind paw RF and located in the medial part of the deep layers of the dorsal horn (752 ± 27 μm; n = 12). Note that 25% of them were found to project to supraspinal structures (Juif and Poisbeau, 2013). After stimulation of the RF, the recorded neuron emitted APs. Two protocols of stimulation were done: (i) 60 stimulations at a frequency of 0.2 Hz (*i.e.* 5 min of recording); stimulus intensity of 1.5 times C-fiber threshold; pulse duration of 1 ms and (ii) wind up (30 stimulations, frequency: 1 Hz, intensity: 3xC-fiber threshold, pulse duration: 1 ms). Wind up efficiency was assessed through the slope and was calculated as a ratio (number of action potentials emitted by the neuron after last stimulation and divided by the number of APs triggered by the first stimulation).

Post-stimulus histograms were built by counting the number of APs corresponding to the activation of fast-conducting Aβ (delay to stimulus artifact <20 ms), slow-conducting Aδ (delay of 20–90 ms) and very slow-conducting C fibers (delay of 90–300 ms), as it is described in the literature for rats weighting about 250 g or more and used for electrophysiology experiments (Urch and Dickenson, 2003). With such animals, APs observed 300–800 ms after the stimulus artifact were considered as being part of the postdischarge. For this experimental approach, it is assumed that most of the non-nociceptive and nociceptive informations are mostly transmitted via Aβ fibers and C fibers, respectively in naïve animals. AP changes were compared before and immediately after the topical EFX application on the spinal cord.

2.2. Behavioral testing

All animals were habituated to the room and to the tests at least one week before starting the experiments. Mechanical nociceptive thresholds were measured using a calibrated forceps (Bioseb, Vitrolles, France) as previously (Aouad et al., 2009). Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps are placed at each side of the paw and a gradually increasing force was applied. The pressure, in gram, producing withdrawal of the paw or in some cases the vocalization of the animal, corresponds to the nociceptive threshold value. This manipulation was performed three times for each hindpaw and the values were averaged.

2.3. Drugs and treatments

EFX (Biocodex, Gentilly, France) was prepared in saline (NaCl 0.9% in distilled water) containing 1% tween 80 (v/v; Sigma, St Louis, USA) and was injected intrathecally 20 min before behavioral testing (dose: 0.6 μg in 20 μl). EFX was applied at the surface of the spinal cord during in vivo electrophysiological recording at 3 different concentrations: 5, 30 and 60 μM. Bicuculline (Sigma–Aldrich, France) was diluted in saline and administered on the spinal cord at a final steady-state concentration of 10 μM. Analysis was performed 15 min after EFX application. After this period, EFX was washed out from the saline solution covering the exposed spinal cord segment.

At the end of the study, EFX was also tested using in vivo electrophysiology of WDR neurons on monoarthritic rats, one week after a unilateral knee injection of 50 μl CFA (complete Freund's adjuvant; Sigma St Louis, MO, USA). The control animals received an equivalent volume of mineral oil, the vehicle of CFA, as previously published (Aouad et al., 2014b).

2.4. Statistics

All data are expressed as mean ± standard error of the mean (SEM). Repeated measure one-way ANOVA followed by Bonferroni comparisons was used to analyze the effects on AP firing while recording from spinal neurons in vivo. Student's t test helped compare the electrical thresholds between two groups (unpaired) or before/after drug application (paired). When parametric tests were inappropriate (low N number of observations or data not normally distributed, A Kruskal–Wallis test or Wilcoxon matched pair test was used. In this later case, Dunn's multiple comparisons posthoc test was used to compare the experimental value to the control. Differences were considered to be statistically significant for $p < 0.05$.

3. Results

3.1. Etifoxine increases mechanical nociceptive threshold and reduces the excitability of WDR neurons after peripheral nociceptive stimulation

In freely-moving animals, mean mechanical thresholds were of 583.3 ± 19.7 g (n = 7) and they remained unchanged after injection of the vehicle of EFX (Fig. 1B; 542.4 ± 14.3 g; n = 5). In sharp contrast, EFX injection resulted in a significant increase of mechanical threshold, which reached a mean value of 768.1 ± 22.2 g (n = 5; Wilcoxon, $p < 0.05$). Compared to control, this corresponded to an increase by about 42% thus confirming the acute anti-nociceptive properties of EFX when spinally administered.

To further analyze EFX action on spinal nociceptive processing, we recorded WDR neurons and first characterized its possible effect of the electrical activation threshold required to observe an A and C-mediated action potential (AP) discharge in WDR neurons (representative traces in Fig. 1A). Compared to control (i.e. before application), we failed to see any changes in the thresholds while using concentration of 5 μM (Fig. 1C). When compared to control (basal in Fig. 1C), this increase was particularly robust after application of EFX at 30 μM (Aβ: from 4.3 ± 0.3 V to 6.3 ± 0.5 V, n = 8; one-way Anova, $F_{4,40} = 17.83$, Bonferroni $p < 0.01$; C: from 21.7 ± 0.4 V to 26.4 ± 0.3 V, n = 12; Bonferroni $p < 0.001$) and at 60 μM (Aβ: 7.4 ± 0.3 V, Bonferroni $p < 0.001$, Aδ: from 15.0 ± 1.2 V to 23.2 ± 0.6 V, n = 5; Kruskal Wallis, $p < 0.01$, Dunn's comparisons $p < 0.05$ and C: 32.1 ± 0.6 V; Bonferroni $p < 0.001$). This increase was fully abolished when EFX (60 μM) was co-administered with bicuculline (10 μM) confirming that this change was mediated by $GABA_A$ receptors.

EFX effects on spinal nociceptive processing were next characterized by quantifying changes in the number of APs emitted by WDR with respect to the respective contribution of Aβ, Aδ and C sensory neurons. Using a stimulation intensity of 1.5 times the threshold for C fibers (frequency: 0.2 Hz; pulse duration: 1 ms), we only observed a significant decrease in the number of APs after

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